THE INHALATION TOXICITY OF SARIN (GB) VAPOR IN RATS AS A FUNCTION OF EQUILIBRATION TIME FOR TEN MINUTE EXPOSURES

J. Steven Anthony, Mark V. Haley, James H. Manthei, Ruth A. Way, David C. Burnett, Bernardita P. Gaviola, Douglas R. Sommerville, Ronald B. Crosier, Robert J. Mioduszewski, Sandra A. Thomson
Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD 21010-5424

Charles L. Crouse, Kathy L. Matson
GEO-CENTERS, INC., Abingdon, MD 21009

ABSTRACT

Characterizing the toxicity of airborne exposures to chemical warfare agents requires sensitive, accurate and reliable analytical chemistry methods as well as adequate methods for generating and controlling the test atmosphere. In addition to concentration and exposure time, it is necessary to examine what effect the chamber equilibration time (t_{99}) has on measured biological endpoints for shorter duration inhalation exposures (i.e. $\leq$ 10 minutes). The t_{99} is defined as the time necessary for an exposure chamber to reach 99% of its experimental concentration. Once this value is reached, the chamber concentration will not rise more than an additional 1%, regardless of exposure time. MacFarland (1975) has suggested that for short-term dynamic exposures, exposure time (t) should be greater than (or equal to) 13(t_{99}) (MacFarland's Rule). Although there is no problem in adhering to this guideline for longer exposures (e.g. 60 or 240 min), adherence is not practical for exposures of 10 min or less where chamber dynamics will only allow the t_{99} value be kept to a minimum. The present study examined dose-response (lethality) relationships for GB vapor in rats derived from 10-minute exposures with t_{99} values that do not adhere to MacFarland's Rule, i.e., 2.1, 5.2, or 8 minutes. It was generally concluded that differences in LCt_{50} values collected under the above t_{99} conditions were minimal and would not be considered statistically significant.

INTRODUCTION

Traditional predictions of GB dosage-mortality relationships over time using Haber's rule[1] have not been supported by the results of experimental studies involving exposure durations up to six hours.[2,3] An inverse linear relationship between concentration (C) and time (t), as implied by Haber's Law, does not exist in these situations. Mioduszewski *et al*., (2001) examined the dose-response effects of Sarin (GB) vapor for lethality in rats at various exposure durations up to six hours. It was found that the assumption regarding the relationship between exposure dose and lethality used historically (Haber's rule; Haber, 1924) to predict CW agent toxicity was not adequate to describe the lethal response data over time. For many acutely toxic gases and aerosols, toxic effects cannot be adequately related to the Ct product.[4,5] For these materials, the influence of concentration is usually more pronounced than that of exposure time. In

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 OCT 2005	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
The Inhalation Toxicity Of Sarin (Gb) Vapor In Rats As A Function Of Equilibration Time For Ten Minute Exposures	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD 21010-5424**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001851, Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research, 17-20 November 2003.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**11**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

other words, a high concentration for a short period has a more severe effect than a low concentration for a longer time, given the same Ct.

In addition to concentration and exposure time, it is necessary to examine what effect the chamber equilibration time (t_{99}) has on measured biological endpoints for shorter duration exposures (i.e. $\leq$ 10 minutes). Toxicologists define t_{99} as the time necessary for an exposure chamber to reach 99% of its experimental concentration.[6] Once this value is reached, the chamber concentration will not rise more than an additional 1%, regardless of exposure time.[6] During inhalation tests, the time necessary to reach t_{99} is theoretically the same as the time necessary for chamber decay or "outgassing".[7] For short term exposures, it has been suggested that dynamic systems should adhere to MacFarland's Rule. This rule states that the exposure time (t) should be greater than (or equal to) $13(t_{99})$. It is generally believed that inaccurate Ct values are delivered if this relationship is not followed.[8] Longer equilibration times and decay times are deemed responsible for these errors. However, no experimental data were found in the literature that proved or disproved this hypothesis.

For exposures, such as 60 or 240 minutes, adherence to this guideline is possible, but shorter exposures will not allow for strict adherence to this rule. The t_{99} value may only be kept at a minimal value of which the dynamics of the inhalation chamber will allow. Airflow and chamber size are the variables present in the t_{99} equation which impart these limitations. High airflows will reduce the t_{99} but the distribution through the chamber will be affected and the concentration will no longer be uniform. If the volume of the chamber is too small, enough space will not be available to expose the animals. The objective of the present study is to develop separate LCt_{50} curves for 10 minute exposures that possess t_{99} values that do not adhere to MacFarland's Rule. T_{99} values of 2.1,5.2, or 8 minutes were used and comparisons were made between the curves to determine whether any observable differences exist in lethality.

MATERIALS AND METHODS

CHEMICALS

Isopropyl methylphosphonofluoridate (sarin, GB) was used in all vapor exposures throughout the study. According to an established method, seven ^{31}P NMR analyses were performed to certify the purity of the test material.[9] GB was found to be 97.3 ± 0.5 wt % pure. No impurity peaks were detected in the phosphorus spectra. A high purity grade of triethylphosphate (>99.9% TEP; Aldrich Cat. No. 24,089-3) was used as the internal standard for the GB purity assays. External calibration standards were prepared using high purity grade hexane solvent (purity> 87.7% n-hexane, >99.9% n-hexane and isomers; supplier: Burdick and Jackson).

VAPOR GENERATION

The vapor generation system was located at the chamber inlet and was contained within a stainless steel glove box maintained under negative pressure. A gas-tight syringe, containing the test material, was secured into a variable rate, pulse-free syringe drive with the material delivered into a spray atomizer. A syringe needle (stainless steel, 26 gauge, 3" length) was used in the spray atomizer for all GB exposures. Liquid GB entered the top of the sprayer, and mixed with compressed air (30 psi) at 12 L/min. The compressed air broke the liquid into fine droplets and facilitated vapor formation.

Concentration uniformity was checked at several locations throughout the chamber, including areas directly above the animal cages and inside the animal cages. At higher generated agent concentrations,

vacuum pumps drew air through glass fiber filter pads at high flow rates to assure the absence of aerosols. Subsequent analyses showed that no agent aerosol was present.

SAMPLING AND MONITORING EXPOSURE CHAMBER FOR GB VAPOR

The 750-liter dynamic whole-body exposure chamber (Figure 1) was located in the middle of a 20,000-liter containment chamber. The exposure chamber was hexagonal and constructed of stainless steel. Plexiglas windows that ran the length of each side permitted observation of toxic signs in the rats during exposure runs. The interior of the exposure chamber was maintained under negative pressure as recorded by a calibrated magnahelix (0-1" water). Room air was drawn through the exposure chamber (400-1700 L/min) and measured at the chamber outlet with a calibrated thermo-anemometer. The rotation speed of the exposure chamber fan (in revolutions per minute) was also monitored as a check for airflow readings. Temperature and humidity were recorded for every exposure.

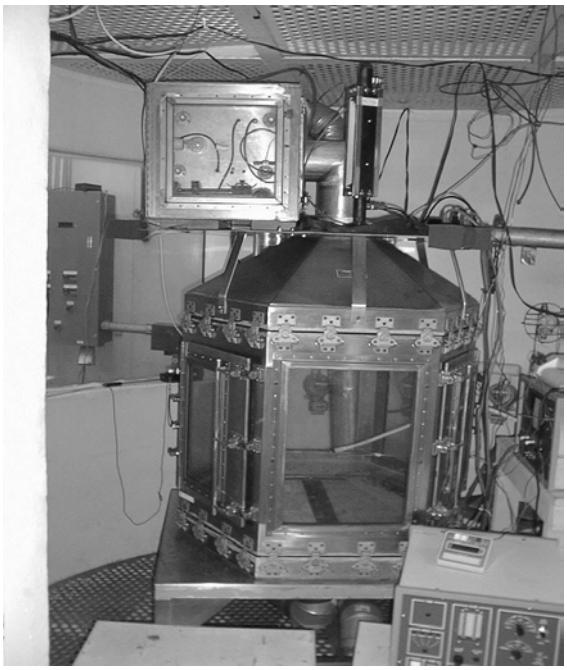

Figure 1. 750-liter experimental exposure chamber system.

THERMAL DESORPTION SOLID SORBENT TUBE

The thermal desorption solid sorbent tube system consisted of a heated transfer line, heated external switching valve, thermal desorption unit, and a gas chromatograph with flame ionization detection. Samples were drawn from the middle of the exposure chamber through a six-foot silica transfer line (1/16″ o.d. x 0.004″ i.d.) and held at 150 ^{o}C. Flow rates (measured before and after sampling) were either 20 ml/min or 40 ml/min, and sampling times were either 1, 4, or 5 minutes, depending on chamber concentration. The sample entered a heated six-port gas-switching valve before depositing onto a Tenax-TA sorbent tube. The solid sorbent material was used to trap the vapor, concentrate it, and inject it directly onto a gas chromatograph-flame ionization detector (GC-FID) for subsequent detection and quantitation. External standards were injected into the end of the transfer line to simulate identical collection conditions between standards and samples. Generated calibration curves were used to calculate

chamber concentrations. To increase the accuracy of experimental concentrations, samples were continually drawn during the exposures as often as the experimental sampling cycle would allow.

PHOSPHORUS MONITOR (HYFED)

Real-time monitoring of chamber concentration was performed with a phosphorus analyzer (HYFED, Model PH262, Columbia Scientific, Austin, TX). Output of the analyzer was recorded on a dual channel strip chart recorder depicting the concentration profile (rise, equilibrium, and decay) of the chamber along with stability of concentration during the exposure time. The rise in concentration, or chamber equilibration time, is dependent on various conditions with airflow through the chamber being the most dominant. Chamber sampling was only performed during the chamber equilibrium phase. Following the 10-minute purge time, both the HYFED response and recorder output returned to baseline, indicating that the chamber was sufficiently purged.

ANIMAL EXPOSURES

ANIMAL MODEL

Young adult male and female Sprague-Dawley rats (8-10 weeks) were obtained from Charles River Laboratories, Inc., Wilmington, MA. The animals were identified by tattoo on the tail, segregated according to sex and housed individually in plastic shoebox cages. They were placed on racks in an American Association for Accreditation of Laboratory Animal Care (AAALAC) accredited facility (Bldg. E-3150). The animals were housed for a minimum of 3 days of quarantine and for the post-exposure period (14 days). Ambient conditions were maintained at 70 ± 5^{O} F, 30 - 70% relative humidity, and a 12:12 hour light-dark cycle. Rats were provided with certified laboratory rat chow and filtered house water *ad libitum*, except during exposure.

WHOLE –BODY INHALATION EXPOSURES

Prior to exposure, animals were placed in two compartmentalized cages (20" x 14" x 4"), each able to hold 10 rats. All rats served as their own controls. As in Mioduszewski, *et al* (2001) and (2002), same gender rats were arranged on alternating diagonals within the two cages. Rats were exposed (whole-body) for 10 minutes to a fixed concentration of GB vapor for one of three t_{99} times (2.1, 5.2,or 8 minutes). During chamber operations, the airflow through the chamber was kept constant.

Due to differences in sensitivity to GB between genders, it was not always desirable to expose both sexes simultaneously to a particular concentration. Certain concentrations might result in an all or none outcome for one gender. Therefore, the sexes were occasionally exposed to different concentrations for a given exposure duration.

Lethality and sub-lethal clinical signs (e.g., miosis, convulsions, tremors, salivation, prostration, and labored breathing) were monitored (from an observation point outside of the exposure chamber) during and after exposure (within the first hour post-exposure and once daily for up to 14 days). A statistical package, version 13 of MINITAB® (Minitab, Inc., State College, PA), was used to perform probit analyses of the lethality data.[10]

RESULTS/DISCUSSION

FORMULATING AN EMPIRICAL LETHALITY PROBABILITY MODEL FOR THE RAT

Using binary logistic regression with a normit link function, the following linear relationship was established. For the probability of lethality, let Y = normit (where normit = probit –5). Logarithms are base 10.

24-HOUR DATA (EQUATION 1)

$Y = -28.416 + 11.026$ (if male) $+ 20.891*\text{LogC} - 9.166*\text{LogC}$ (if male) $- 0.12255*t_{99}$
where t_{99} is in minutes and base 10 logs of concentration in mg/m^3 are used

This reduces to:

$Y = -28.416 + 20.891*\text{LogC} - 0.12255*t_{99}$ (for females)

$Y = = -17.390 + 11.725*\text{LogC} - 0.12255*t_{99}$ (for males)

LCT_{50} CURVES FROM EMPIRICAL LETHALITY PROBABILITY MODEL

Predicted male and female LC_{50} relationships from Equation 1 are shown in Figure 2. The squares and diamonds on the graph represent the male and female LC_{50} values, respectively, as determined from individual probit analyses, with the actual values listed in Table 1 (along with the corresponding probit slopes). Vertical lines in Figure 2 represent the 95% fiducial limits for the individual LC_{50} values. The dots are the LC_{50}'s from separate probit analyses at each t_{99} and sex; the fiducial intervals come from these probit analyses. The lines indicating log(LC_{50}) as a function of t_{99} come from a binary regression of mortality on log(concentration), sex, t_{99}, and sex*log(concentration). As discussed by Slob (2002), the slight differences in LC50 values are likely caused by systematic differences between the exposure groups.[11] These may be deemed minimal within the constraints of experimental/animal variability.

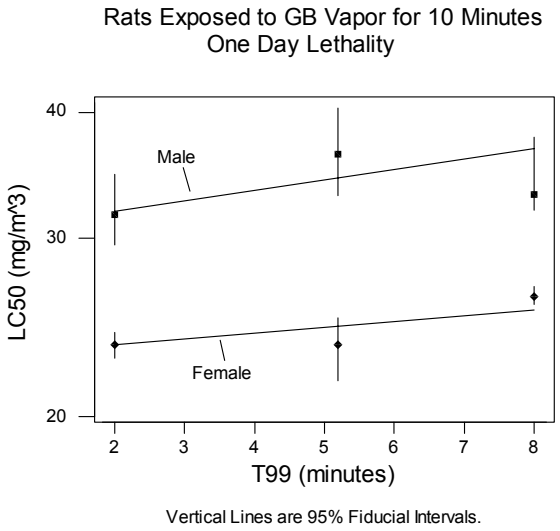

Figure 2. Lethality within 24 hours from GB vapor (Vertical Bars Are 95% Fiducial Limits).

Table 1. LC_{50}, $LCt_{50,}$ 95% Fiducial Intervals, and Slopes for GB Vapor-Induced Lethality (24 hours Post-Exposure).

t_{99} (min)	Females					Males				
	LC_{50} (mg/m^3)	LC_{50} 95 %FI (mg/m^3)	LCt_{50} (mg min/m^3)	LCt_{50} 95 %F.I (mg/m^3)	Slope	LC_{50} (mg/m^3)	LC_{50} 95 %F.I (mg/m^3)	LCt_{50} (mg min/m^3)	LCt_{50} 95 %F.I (mg/m^3)	Slope
2	23.5	22.9-24.2	235	229-242	37.9	31.7	29.6-34.8	317	296-348	14.8
5.2	23.6	21.7 – 25.1	236	217-251	15.6	36.4	33.1-40.4	364	331-404	12.5
8	26.3	25.9-26.9	263	259-269	60.4	33.2	32.0-37.8	332	320-378	24.0

CHAMBER PROFILE AND CALCULATION OF t_{99}

An example of a concentration profile for a dynamic inhalation exposure is shown in Figure 3 with the following notation: t_a - the start of the exposure (or when the syringe pump was started); t_b - the point at which the syringe pump is turned off (but the air still left on to clear the chamber); t_{99} - the point at which the chamber has reached 99% of its experimental concentration; and t_c – the point at which the agent has been cleared from the chamber. Animals are not withdrawn from the chamber until t_c is exceeded. The concentration-time profile generated with this type of chamber is described in a review by MacFarland (1987).[12] His definition of exposure duration was the one used in this study: the interval from the start of the flow of agent into the chamber to the time when the agent supply is stopped(t_a to t_b). As described by Silver, an accurate Ct product is delivered in these dynamic systems as the area under the curve representing the exponential rise equals the area representing the exponential decay. If the shaded area from t_b to t_c is fit into the shaded area from t_a to t_{99}, then the "square" function of the Ct exposure is complete. Therefore, Ct may be used to quantify the exposure from t_a to t_b even though there is an area "lost" in ramping up to equilibrium.

Real time monitoring of the chamber concentration is performed during exposures (HYFED) with the output recorded on a strip chart recorder. Figure 4 shows recordings for a 2.1 and 5.2 minute t_{99}. As expected, the rise (t_a to t_{99}) and decay (t_b to t_c) times are longer as the t_{99} time increases. The theoretical t_{99} times may be calculated according to Equation 2.

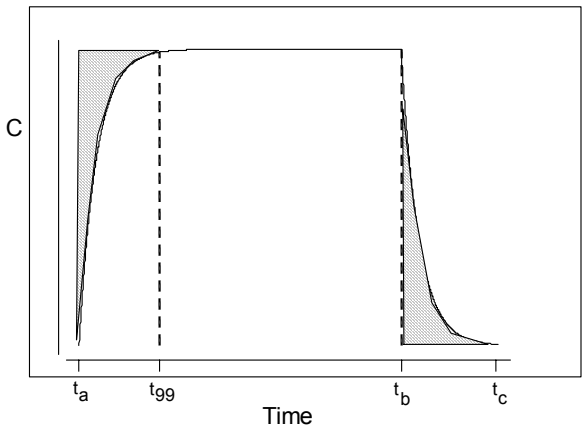

Figure 3 . Theoretical exposure chamber profile.

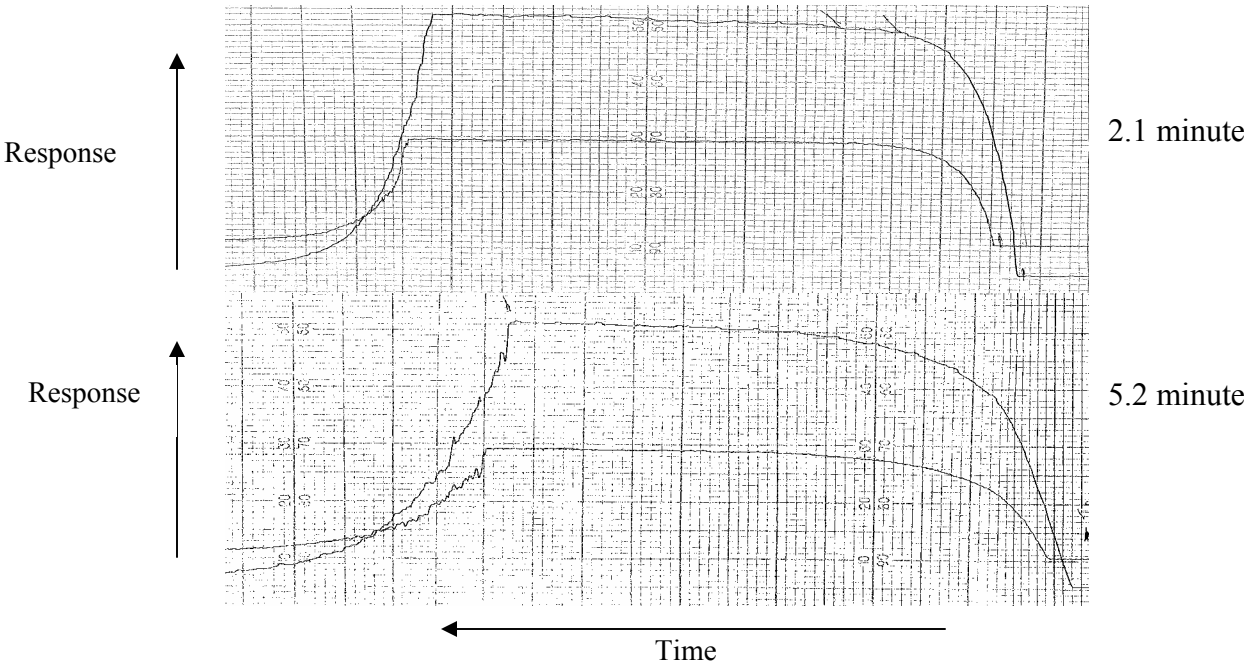

Figure 4. Experimental HYFED readings for 2.1 and 5.2 minute t_{99}.

Equation 2: $C= (w/b)[1-e^{-bt/a}]$ where

C= chamber concentration
w = the weight of the agent introduced per minute
a= the volume of the chamber (for this study, equals 750 liters)
b = the flow rate through the chamber

According to Equation 2, the concentration in the chamber will rise exponentially to some constant value, with the final equilibrium value being solely dependent on the ratio of a to b. However, in order for the preceding equation to be valid for a particular dynamic chamber system, there must exist a uniform distribution of agent vapor throughout the chamber, and w and b must be constant. For the instance of 99% of the equilibrium concentration (in this case t_{99}), Equation 2 may be simplified to:

$$99 = 100[1-e^{-bt/a}]$$
or
$$t_{99} = 4.605\ a/b.$$

A more generic formula would be $t_x = K\ a/b$, where $K = -\ln(1-x/100)$ and ln is the natural (base e) logarithm. The ratio a/b represents the time needed for one air change in the inhalation chamber while K represents the number of these air changes that are necessary to reach the desired percentage of the target concentration – hence 99%. Previous concentration levels in the chamber have no involvement in the calculation for t_{99}. Therefore if the same size chamber is used for all exposures, the only variable to alter t_{99} is airflow.

As experimentally determined by Silver, values of K for complimentary values of x are provided below:

X	K
99	4.605
95	2.996
90	2.303
85	1.897
80	1.609

POTENCY RATIO

Relative GB potencies for varying chamber equilibration times are shown in Table 2. When combining data from male or female exposures, the probit analysis routine uses the same slope for both agents.

Table 2. Relative Potency for GB at different equilibration times – 24-hour data.

Sex	Relative Potency		
	2 min t_{99}/5.2 min t_{99}	2 min t_{99}/8 min t_{99}	5.2 min t_{99}/8 min t_{99}
Males	1.15 (1.04-1.26)	1.07 (0.98-1.19)	0.93 (0.86-1.03)
Females	1.01 (0.96-1.07)	1.12 (1.07-1.18)	1.11 (1.05-1.17)

CONCLUSIONS

These studies assessed the impact of different chamber equilibration times on determining the LC_{50} of rats exposed to an organophosphate vapor. Male LC_{50} values were higher than the respective female LC_{50} values at all three equilibration times tested. The vapor concentration profile for GB in the experimental chamber confirmed that the delivered Ct for rise and exponential decay phases of the exposure were equal. No significant differences in LC_{50} for a ten-minute exposure of GB vapor were found comparing three equilibration times; minimal differences were attributed to experimental/animal variability. Thus, the present findings did not confirm the importance of equilibration time requirements for a short dynamic exposure as proposed by MacFarland's rule.

REFERENCES

1. Haber, F.R., "Zur geschichte des gaskrieges," In Funf Vortrage aus Jahren 1920-1923, Spinger, Berlin,1924.

2. Mioduszewski, R.J.; Manthei, J.H.; Way, R.A.; Burnett, D.C.; Gaviola, B.P.; Muse, W.T. Jr.; Anthony, J.S.; Durst, H.D.; Sommerville, D.R.; Crosier, R.B.; Thomson, S.A.; and Crouse, C.L. *Low Level Operational Toxicology Program: Phase I—Inhalation Toxicity of Sarin Vapor in Rats as a Function of Exposure Concentration and Duration*; ECBC-TR-183; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2001; UNCLASSIFIED Report (AD-A394 372).

3. Mioduszewski, R.J.; Manthei, J.H.; Way, R.A., Burnett, D.C.; Gaviola, B.P.; Muse, W.T. Jr.; Sommerville, D.R.; Crosier, R.B.; and Thomson, S.A. Interaction of Exposure Concentration and Duration in Determining Acute Toxic Effects of Sarin Vapor in Rats. *Toxicological Sciences* **2002**, *66***,** pp. 176-184.

4. Larsen, R.I.; Gardner, D.E.; and Coffin, D.L. An Air Quality Data Analysis System for Interrelating Effects, Standards and Needed Source Reductions: Part 5 (NO_2 Mortality in Mice). *Journal of the Air Pollution Control Association* **1979**, *29*, pp. 133-137.

5. ten Berge, W.F.; Zwart, A.; and Appelman, L.M. Concentration-Time Mortality Response Relationship of Irritant and Systemically Acting Vapours and Gases. *Journal of Hazardous Materials* **1986,** *13*, pp. 301-309.

6. Silver, S.D. Constant flow gassing chambers: Principles influencing design and operation. *J Lab Clin Med* **1946,** *31*, pp.1153-1161.

7. *Concepts in Inhalation Toxicology*; McClennan, R.O.; Henderson, R.F., Eds.; Hemisphere Publishing Corporation: New York, 1989.

8. MacFarland, H.N. Respiratory Toxicology. *Essays in Toxicology*, **1975**, *7*, pp. 121-153.

9. Brickhouse, M.D.; Rees, M.S.; O'Connor, R.J.; Durst, H.D. *Nuclear Magnetic Resonance (NMR) Analysis of G-class and VX Nerve Agents and Reaction Masses Produced by Their Chemical*

Neutralization; ERDEC-TR-449; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 1997; UNCLASSIFIED Report (AD-A339 308).

10. *Probit Analysis*; 3rd ed.; Finney, D.J., Ed.; Cambridge University Press: Cambridge, 1971.

11. Slob, W. Dose-Response Modeling of Continuous Endpoints. *Toxicological Sciences* **2002,** *66,* pp-298-312.

12 *Inhalation Toxicology – Chapter 4 Designs and Operational Characteristics of Inhalation Exposure Equipment* (*MacFarland, H.N);* Salem, H.S., Ed.; Marcel Dekker, Inc: New York, 1987.